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In Vivo and In Vitro Action of New Antibiotics Interfering with the Utilization of N-Acetyl-Glucosamine-N-Acetyl-Muramyl-Pentapeptide

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Recent literature on the antibiotics enduracidin, moenomycin, prasinomycin, and 11.837 RP suggested an interaction with murein synthesis. Incubation of sensitive strains from *Bacillus cereus* and *Staphylococcus aureus* in a "wall medium" containing labeled L-alanine showed that all four antibiotics inhibited the incorporation of alanine into murein and gave rise to accumulation of radioactive uridine diphosphate-N-acetyl-muramyl (UDP-MurNAc)-pentapeptide. Peptidoglycan was synthesized when the particulate enzyme of *B. stearothermophilus* was incubated with the murein precursors UDP-N-acetyl-glucosamine (UDP-GlcNAc) and UDP-MurNAc-pentapeptide. The newly formed polymer was less accessible for lysozyme and more strongly bound to the acceptor than the same product from the *Escherichia coli* particulate enzyme. After incubation in the presence of penicillin, a greater part of the peptidoglycan was lysozyme sensitive and more loosely bound to the acceptor. The antibiotics enduracidin, moenomycin, prasinomycin, and 11.837 RP inhibited peptidoglycan synthesis by the *B. stearothermophilus* particulate enzyme. The rate of synthesis of GlcNAc-MurNAc(-pentapeptide)-P-P-phospholipid was independent from the addition of these antibiotics, but its utilization was strongly inhibited. With the present results, it is not possible to distinguish the mechanisms of action of enduracidin, moenomycin, prasinomycin, and 11.837 RP from the mechanisms of action of vancomycin and ristocetin.

Almost all information concerning the last reactions in murein synthesis has been obtained from in vitro systems using particle bound enzymes (2, 14, 15).

The two cell wall precursors, uridine diphosphate-N-acetyl-muramyl (UDP-MurNAc)-L-ala-D-glu-m-diaminopimelic acid (DAP)-D-ala-D-ala and UDP-N-acetyl-glucosamine (UDP-GlcNAc) are bound to the membrane by a phospholipid carrier in two enzymatic reactions to form a disaccharide (-pentapeptide)-P-P-phospholipid (1, 30). The structure of the phospholipid carrier has been determined to be a C_{55}-isoprenoid alcohol phosphate (9). It transports the disaccharide-pentapeptide to its acceptor. This reaction is inhibited in vitro by vancomycin and ristocetin (1, 15). The lipid carrier is regenerated by dephosphorylation, a reaction which is inhibited by bacitracin (28).

The pentapeptide chain of the new building block may remain free or can be cross-linked to another peptide chain by a transpeptidase, with liberation of D-alanine (14, 15, 31, 35). Penicillins and cephalosporins inhibit the transpeptidase from *Escherichia coli* in vitro (14, 15). In vivo experiments on the action of penicillin in *E. coli* (27) and *Proteus mirabilis* (17), however, did not show a decrease in cross-linkage. In the cell-free system from *E. coli* Y-10, one-half of the D-alanine molecules is liberated from the pentapeptide, whereas the other half is found back in the peptidoglycan (15). This is in good agreement with the analysis of lysozyme-degraded murein sacculi, which shows that mainly tetrapeptides and a small amount of tripeptides are found (33). Besides the transpeptidase, the D-alanine carboxypeptidase I (16) may also play a role in the formation of tetrapeptide from pentapeptide. Both enzymes are sensitive to penicillin in vitro (15, 16). The question as to whether the two activities found in vitro are due to products of different genes has not yet been elucidated.

Recent literature on some new antibiotics has suggested that they might interfere with the envelope-bound steps in murein synthesis. Enduracidin (32) and moenomycin (13) lysed *S. aureus*

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and gave rise to accumulation of UDP-MurNAc-pentapeptide. Prasinomycin, a mixture of several active components (34), caused lysis of the same organism and accumulation of an unidentified sugar nucleotide (18). Research on the structure of moenomycin showed D-glucosamine as one of the sugar components (12) and C25-polyisoprenic alcohols as lipid components (11). This part of the structure may resemble the lipid carrier and, therefore, interfere with its reactions. Like prasinomycin (18), 11.837 RP seems to have structural relationships with moenomycin (21), and it was tested for that reason; however, as far as we know, inhibition of murein synthesis by 11.837 RP has never been shown.

The present paper describes experiments on the influence of these antibiotics on the synthesis of peptidoglycan in vivo and in vitro.

MATERIALS AND METHODS

Bacterial strains. *Bacillus stearothermophilus* (NCTC 10339) and *S. aureus* 524/SC were obtained from P. E. Reynolds. *B. cereus* T was obtained from K. Izaki.

Media. The complex growth medium CGPY and the "cell wall synthesis medium" CWSM-I have been described earlier (E. J. J. Lugtenberg and P. G. de Haan, Antonie van Leeuwenhoek J. Microbiol. Serol, *in press*). When *S. aureus* was incubated in CWSM-I, glycine (100 μg/ml) was added to the medium because this amino acid is a component of the *Staphylococcus* cell wall. CWSM-II medium contained (per liter): Na_2HPO_4 (0.26 g), NH_4Cl (2 g), KCl (4 g), $MgCl_2$ (4 g), Na_2SO_4 (0.15 g), $FeSO_4$ (0.10 g), glucose (2 g), uracil (40 mg), L-glutamic acid (120 mg), L-lysine (500 mg), 2.6-diaminopimelic acid (120 mg), and chloramphenicol (50 mg). The *pH* was adjusted to 7.4.

Buffers. Buffer A contains (per liter): 5×10^{-2} M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride and 10^{-2} M $MgCl_2$ (*p*H 7.8). Buffer B contains (per liter): 1.5 M Tris-hydrochloride and 0.3 M $MgCl_2$ (*p*H 7.8).

Growth of bacteria. Bacteria were grown with aeration in CGPY supplemented with 0.5% glucose. They were incubated at 37 C, with the exception of *B. stearothermophilus*, which was grown at 55 C. Optical density was measured with an Unicam-SP600 spectrophotometer.

Murein synthesis in vivo. Murein synthesis in vivo was carried out as described earlier for *E. coli* (Lugtenberg and de Haan, *in press*). A washed suspension of exponentially growing cells was incubated in CWSM-I, supplemented with ^{14}C-L-alanine and, when *S. aureus* was used, also with glycine (100 μg/ml). Incorporation was determined (i) by counting the acid-precipitable activity or (ii) by chromatography of heat-inactivated samples. After autoradiography, the base spot was cut out and counted. The possibility that besides murein also radioactive teichoic acid was counted by both methods will be discussed later on in this paper.

Preparation of particulate enzyme. An exponentially growing culture (optical density = 0.5) of *Bacillus stearothermophilus* (1,600 ml) was chilled in ice water. The procedure was carried out in the cold. The cells were centrifuged for 5 min in a model 18 centrifuge (Measuring & Scientific Equipment, Ltd.) at 15,000 $\times$ g. The pellet was washed with 80 ml of buffer A and resuspended in 16 ml of the same buffer. One volume of cell suspension was added to one volume of plastic beads (2% Styrol-DVB copolymer, 200 to 400 mesh, 40 to 80 μ; Serva Entwicklungslabor, Heidelberg, Germany). The cells were disintegrated with a homogenizer (Measuring & Scientific Equipment, Ltd.) for 3 min at maximum speed under cooling in an ice-salt mixture. The homogenized sample was filtered through a G-1 glass filter under careful suction. The filter was washed twice with buffer A. The combined filtrates were further treated according to Fig. 1. When indicated, pellets were resuspended with a glass rod in buffer A. The resulting preparations P-3′ and P-4′ were used as the particulate enzyme, after dilution in buffer A if necessary. In later experiments, P-3′ and P-4′ were not separated further because the difference in activity between larger and smaller particles was very small. Undiluted enzyme preparations contained about 9 mg of protein per ml.

Cell-free peptidoglycan synthesis. The incubation mixtures contained: (i) 5 μliters of labeled precursor, either UDP-GlcNAc-^{14}C (specific activity, 42 μCi/μmole, 0.05 μmole/ml) or UDP-MurNAc-pentapeptide (specific activity, 4.1 μCi/μmole, 0.32 μmole/ml); the latter compound was labeled with ^{14}C-alanine; (ii) 5 μliters of unlabeled precursor (2 μmole/ml); (iii) 5 μliters of buffer B; (iv) 5 μliters of antibiotic or distilled water; (v) 10 μliters of particulate enzyme. The different components were mixed in the cold and incubated in a water bath.

To obtain rapid information on the activity of the system, the perchloric acid-precipitable activity was determined as described previously (Lugtenberg and de Haan, *in press*). In addition to peptidoglycan the precipitate probably also contains the lipid intermediates.

When detailed information was desired, the incubation mixture was heated for 1 min in a boiling water bath and cooled in ice water. The sample was applied as a 1.5-cm streak to Whatman no. 1 chromatography paper. Chromatography and autoradiography (usually for 4 weeks) were carried out as described earlier (Lugtenberg and de Haan, *in press*). Control tubes did not contain the unlabeled precursor or were inactivated at zero time.

Lysozyme degradation. After incubation of the mixture for peptidoglycan synthesis, the particulate enzyme was heat inactivated. Lysozyme (250 μg/ml) was added, and the mixture was incubated at 37 C for 16 hr. After heat inactivation, chromatography was carried out as described earlier (Lugtenberg and de Haan, *in press*).

Solubility of material synthesized in vitro. After incubation and heat inactivation, the mixture was centrifuged for 10 min at low speed. The pellet was resuspended in sixfold diluted buffer B and centrifuged again. The pellet and both supernatant fluids were chromatographed. Spots, located by autoradiography were cut out and counted as described before (Lugtenberg and de Haan, *in press*).

Protein. Protein was determined according to Lowry et al. (20).

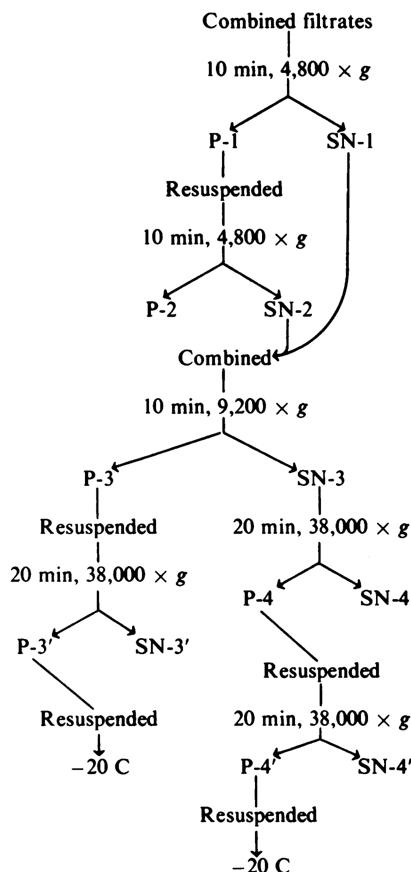

FIG. 1. *Preparation of particulate enzyme. Symbols: P, pellet. SN, supernatant fluid.*

Radiochemicals. L-Alanine-U-^{14}C (specific activity, 156 mCi/mmole) was obtained from Radiochemical Centre, Amersham, England. UDP-GlcNAc-^{14}C (specific activity, 42 mCi/mmole) was obtained from New England Nuclear, Frankfurt a/M, Germany. Radioactive UDP-MurNAc-L-ala-D-glu-m-DAP-D-ala-D-ala was isolated from *B. cereus* T. Cells were grown in 2 liters of CGPY, supplemented with 0.5% glucose to half the optical density of an overnight culture. After centrifugation, they were resuspended into 400 ml of CWSM-II, supplemented with 12.5 μg of vancomycin/ml and 100 μCi of L-alanine-U-^{14}C and incubated under aeration for 40 min at 37 C. The cells were harvested and resuspended into 15 ml of distilled water. The suspension was sonically treated, heated for 10 min in a boiling-water bath, and cooled; 40% trichloroacetic acid was then added to a final concentration of 5%. After centrifugation, the supernatant fluid was extracted three times with 1 volume of ether to remove the trichloroacetic acid. The water phase was neutralized with 1 N NaOH, and the residual ether was removed in a 37 C water bath. The extract was concentrated under reduced pressure to 5 ml. The nucleotide was applied to a Dowex-1 column (200 to 400 mesh) and eluted with a linear gradient from 0 to 0.3 M NaCl in 10^{-2} M HCl. UDP-MurNAc-pentapeptide was located by testing ultraviolet (UV)-positive peaks for radioactivity. The active fractions were combined, neutralized, partly evaporated, and desalted on a Sephadex G-10 column. The UV-positive peak contained 55% of the input activity; it was applied to washed Whatman 3MM paper and chromatographed in isobutyric acid-1 M ammonia (5:3, v/v) for 72 hr. The acetone-washed dry chromatogram showed four UV bands, one of which contained the radioactivity. It was eluted overnight with water and rechromatographed in ethanol-1 M ammonium acetate, *p*H 7.2 (5:2). The UV spectrum of the eluted radioactive material was the same as that of UDP. Amino acid analysis of a sample of the hydrolyzed radioactive band showed that the three amino acids and muramic acid were present in the expected ratio. No impurities were detected.

The modified Morgan-Elson test (24) showed a yield of 8.4 μmoles, which was in good agreement with the UV adsorption test. The specific activity was 4.1 mCi/mmole. The UDP-MurNAc-pentapeptide was stored at −20 C. For unknown reasons decomposition occurred within a few weeks, which happened again after repurification.

Other chemicals and antibiotics. UDP-GlcNAc was obtained from Sigma Chemical Co., St. Louis, Mo. Unlabeled UDP-MurNAc-pentapeptide was prepared in the same way as the labeled form, except that the following modifications were used. Instead of labeled L-alanine, the medium contained 50 mg of unlabeled L-alanine per liter. The column fractions containing cell wall precursors were located by the modified Morgan-Elson test (24). The main positive peak was identified as UDP-MurNAc-pentapeptide. Enduracidin, moenomycin, prasinomycin, and 11.837 RP were gifts from H. Nawa (Takeda Chemical Industries Ltd, Osaka, Japan), G. Huber (Farbwerke Hoechst A. G., Frankfurt a/M, Germany), F. L. Weisenborn (The Squibb Institute, New Brunswick, N.J.) and D. Mancy (Rhône-Poulenc, Vitry sur Seine, France), respectively. The origin of the other chemicals has been described in a previous paper (Lugtenberg and de Haan, *in press*).

RESULTS

Effects of antibiotics on cell wall synthesis in vivo. These experiments were carried out as previously described. The incorporation of ^{14}C-L-alanine in *B. cereus* and *S. aureus* was extremely good. The influence of the four new antibiotics previously mentioned on murein synthesis was studied in *B. cereus*. Prasinomycin, tested in concentrations up to 60 μg/ml, had no effect. Enduracidin, moenomycin, and 11.837 RP in concentrations of 10 μg/ml caused a decrease in the acid-precipitable activity (Fig. 2). When, after 60 min of incubation, a heat-inactivated sample was chromatographed, autoradiograms showed the same spots as were seen for *E. coli* (Lugtenberg and de Haan, *in press*), except that only one precursor spot was observed, which corresponded with UDP-MurNAc-pentapeptide. The activity at the origin was decreased when the cells were incubated with the antibiotics. The ac-

tivities of the precursor were enormously increased: 6, 7, and 22 times by moenomycin, 11.837 RP, and enduracidin, respectively. The chromatographic mobilities of the accumulated precursors were the same, suggesting that 11.837 RP, as moenomycin (13) and enduracidin (32), accumulates UDP-MurNAc-pentapeptide.

Although prasinomycin had no effect on *B. cereus*, it severely inhibited ^{14}C-L-alanine incorporation in the acid-precipitable activity of *S. aureus*. Inhibition was already very strong when the antibiotic concentration was 2 μg/ml. Autoradiograms of chromatographed heat-inactivated samples in CWSM-I showed the same spots as were seen with *E. coli* (Lugtenberg and de Haan, *in press*), except that only one precursor was observed, which had the same chromatographic behavior as lysine-containing UDP-MurNAc-pentapeptide. In the presence of prasinomycin, we observed a strong decrease on the basis spot activity (without antibiotic, 6,050 counts/min; with prasinomycin, 1,670 counts/min) and an enormous increase of activity in UDP-MurNAc-pentapeptide (without antibiotic, 2,290 counts/min; in the presence of prasinomycin, 13,030 counts/min.

Recently Hughes et al. (10) studied cell wall thickening in *B. subtilis* and found that the mucopeptide fraction contained at least three times more radioactivity derived from alanine than the teichoic acid fraction. Therefore, it is likely that the activity we found at the origin of the chromatograms is mainly located in murein; however, because D-alanine is a component of most glycerol-teichoic acids (3), we cannot exclude the possibility that part of the activity is due to D-alanine-containing teichoic acids.

The experiments described above prove that all four antibiotics inhibit cell wall synthesis in vivo and strongly suggest that they give rise to accumulation of UDP-MurNAc-pentapeptide. Thus, the synthesis of cell wall precursors is probably not inhibited by the antibiotics. The target of their action must, therefore, be one of the later steps in murein synthesis. The mechanism of action of the antibiotics was studied in vitro.

Particulate system for peptidoglycan synthesis from Bacillus stearothermophilus. The particulate enzyme from *B. stearothermophilus*, prepared as previously described, was very active. The optimal temperature for peptidoglycan synthesis was between 40 and 50 C (Fig. 3), which is lower than the optimal temperature for growth (60 to 65 C). However, all further experiments were carried out at 37 C although the optimal temperature was somewhat higher.

When labeled UDP-GlcNAc was used as the radioactive substrate, the autoradiogram (Fig. 4)

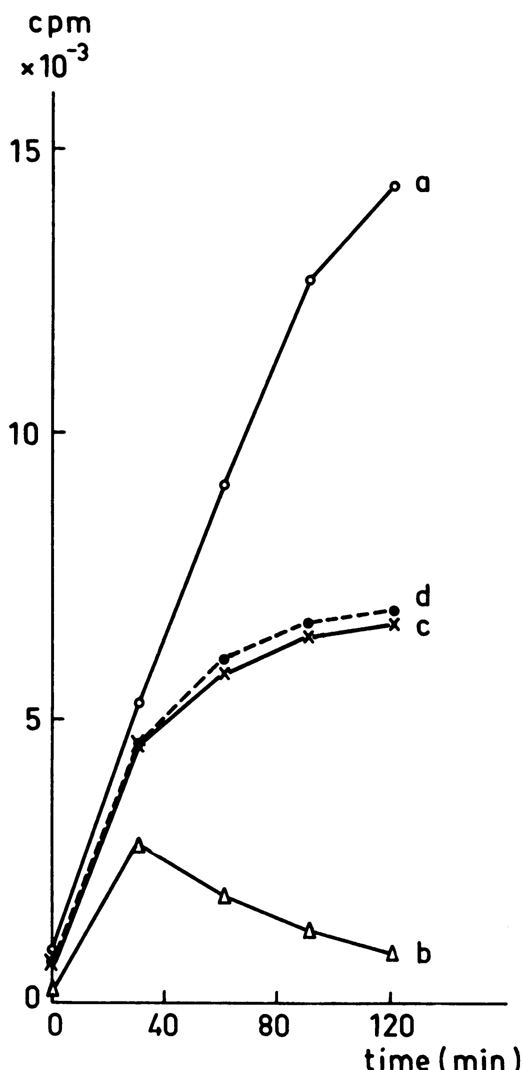

FIG. 2. *Influence of antibiotics on incorporation of ^{14}C-L-alanine in perchloric acid-precipitable material. Exponentially growing Bacillus cereus cells were washed and resuspended in CWSM-I at 37 C. After 15 min of incubation, ^{14}C-L-alanine was added in a final concentration of 1 μCi/ml (zero time), immediately followed by transfer of 2-ml samples to universals containing (a) no antibiotic or (b) 20 μg of enduracidin, (c) moenomycin, and (d) 11.837 RP. At indicated times, perchloric acid-precipitable activity in 0.2-ml samples was determined.*

showed spots from peptidoglycan (R_F 0.0), UDP-GlcNAc (R_F 0.17), and lipid (R_F 0.9). A component with R_F 0.33 was also detected and was identified as an impurity in UDP-GlcNAc. When ^{14}C-alanine-containing UDP-MurNAc-pentapeptide was used as the radioactive sub-

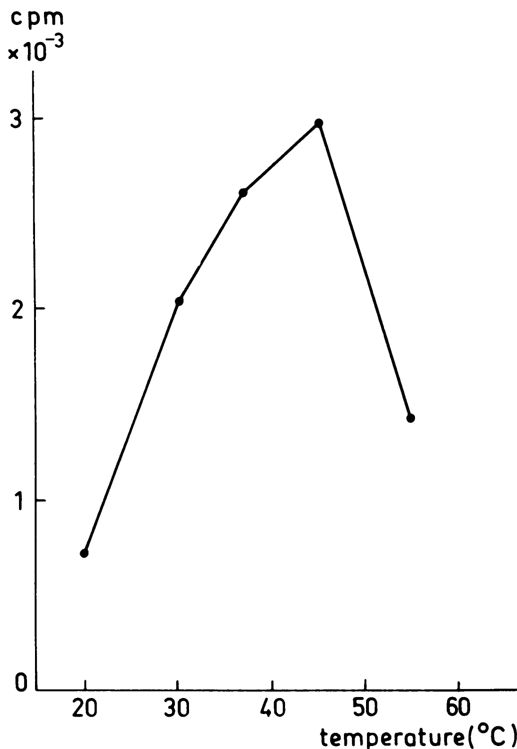

FIG. 3. *Temperature dependance of peptidoglycan synthesis by the particulate Bacillus stearothermophilus enzyme. Incubation mixtures containing labeled UDP-GlcNAc were incubated for 20 min at 37 C, heated, and chromatographed. After autoradiography, the origin was cut out. The plotted values are corrected for the activity from a complete incubation mixture, inactivated at zero time.*

strate, peptidoglycan, the labeled precursor (sometimes with its degradation products), alanine (R_F 0.6), and lipid intermediates were detected.

Adenosine triphosphate (ATP) added to the incubation mixture to a final concentration of 0.25 μmole/ml did not stimulate the peptidoglycan synthesis. Stimulation by ATP was found for the *E. coli* particulate enzyme by Araki et al. (2).

To show that the activity at the origin of the chromatogram was due to peptidoglycan, two complete mixtures with labeled UDP-GlcNAc were incubated and heat inactivated. One mixture was treated with lysozyme. After chromatography, the base spot of the latter mixture contained 67.5% less activity than the untreated control. The solubilized activity was found at higher R_F values.

When penicillin G (300 μg/ml) was included in the incubation mixture, almost the same activity (82.5%) was found at the basis as when penicillin was absent. However, 91.5% of this material was lysozyme degradable. The results of these experiments are summarized in Table 1.

Izaki et al. (15), working with the particulate system from *E. coli*, found considerable degradation of peptidoglycan, especially when formed in the presence of penicillin. In the *B. stearothermophilus* particulate system, we did not find degradation products, even when the incubation

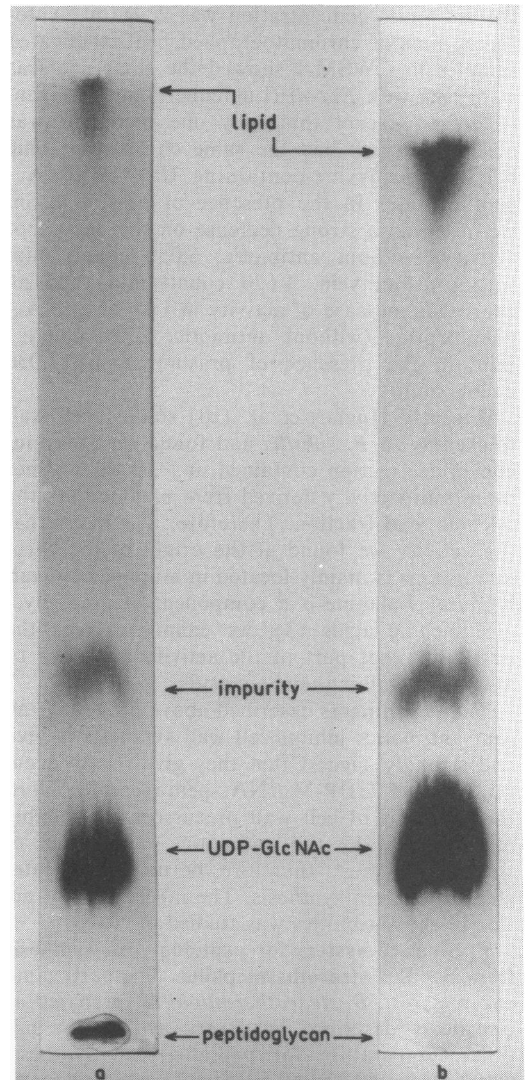

FIG. 4. *Inhibition of peptidoglycan synthesis in vitro. Bacillus stearothermophilus particulate enzyme was diluted to 2.4 mg of protein/ml before addition. P-3' and P-4' were not separated. Radioactive UDP-GlcNAc was used. Incubations (a) without and (b) with enduracidin (20 μg/ml, final concentration) were carried out at 37 C for 40 min.*

TABLE 1. *Solubility in water and accessibility to lysozyme of peptidoglycan synthesized by Bacillus stearothermophilus particulate enzyme in the absence and presence of penicillin*[a]

Incubation mixture	Treatment	Measured sample	Activity (counts/min) in peptidoglycan (R_F 0.0)
1 No UDP-MurNAc-pentapeptide		Total	151
2 Complete		Total	6,277
Complete	Centrifugation	Washed pellet	5,149
Complete	Centrifugation	Supernatant fluids	488
Complete	Lysozyme	Total	2,028
3 Complete + penicillin		Total	5,088
Complete + penicillin	Centrifugation	Washed pellet	3,481
Complete + penicillin	Centrifugation	Supernatant fluids	1,687
Complete + penicillin	Lysozyme	Total	434

[a] Three tubes containing (1) complete mixture lacking uridine diphosphate-*N*-acetyl-muramyl (UDP-MurNAc)-pentapeptide, (2) complete mixture, and (3) complete mixture + 300 μg of penicillin G/ml were incubated during 40 min at 37 C. All mixtures contained UDP-GlcNAc-^{14}C and a final protein concentration of 1 mg/ml. Inactivation, centrifugation, lysozyme treatment, chromatography, and autoradiography were carried out as described in the text and by Lugtenberg and de Haan (*in press*).

mixture contained penicillin. To explain their results, Izaki et al. (15) supposed that peptidoglycan synthesized in the presence of penicillin was more soluble and, therefore, better accessible for endogenous autolytic enzymes; this was proved by finding more peptidoglycan in the supernatant fluid when it was synthesized in the presence of penicillin (15). In their system, lysozyme degraded all of the peptidoglycan formed in the absence of penicillin (15). In the *B. stearothermophilus* particulate system, 32.5% of the synthesized material could not be degraded by lysozyme. The undegradable material consisted of only 8.5% when penicillin was present (Table 1). For this reason and because hardly any material was synthesized when the unlabeled precursor was omitted, we believe that the material at the origin of the chromatogram is peptidoglycan.

Izaki et al. observed that peptidoglycan synthesized in the presence of penicillin spreads on a chromatogram (14, 15). Their "spreading" product was almost completely soluble in water (80 to 90%), whereas the peptidoglycan formed in the complete system without additions was less water soluble (50 to 75%; 15). Because the observation of a spreading product is not quantitative and highly dependent on the way in which the sample is applied to the chromatogram (J. L. Strominger, *personal communication*), we preferred to test the solubility in water. Experiments were carried out as previously described. The results are given in Table 1. Only 9% of the peptidoglycan synthesized in the absence of penicillin was found in the supernatant fluid, whereas 31% was water soluble when synthesized in the presence of penicillin.

Reynolds (26) reported that the activity of the *B. stearothermophilus* system was higher than the activities of other described systems. Our results, presented above, showed that peptidoglycan synthesized by the *B. stearothermophilus* system could not be completely degraded by lysozyme and was almost insoluble in water. Reynolds and our results show that the *Bacillus* system simulates the in vivo situation better than the *E. coli* system.

A time course of the complete system showed that the activity from the precursor appears first in the lipid fraction and finally in peptidoglycan, no matter which labeled precursor was used. The results of the experiment with UDP-GlcNAc-^{14}C are presented in Fig. 5.

In a particulate system of *E. coli* Y-10, in which the other precursor was labeled, Izaki et al. (15) found the same results, except that the relative activity in the lipid fraction was many times higher.

Action of antibiotics on peptidoglycan synthesis in vitro. Preliminary experiments, with UDP-MurNAc-pentapeptide as the radioactive substrate, indicated that enduracidin, moenomycin, and 11.837 RP inhibited cell-free peptidoglycan synthesis and accumulated radioactivity in the lipid fraction (Table 2). Bacitracin, known as an inhibitor of cell wall synthesis, gave a very strong inhibition of peptidoglycan synthesis, but no accumulation of lipid-intermediates (Table 2), as was expected from its mechanism of action (28).

To test whether the utilization of MurNAc-

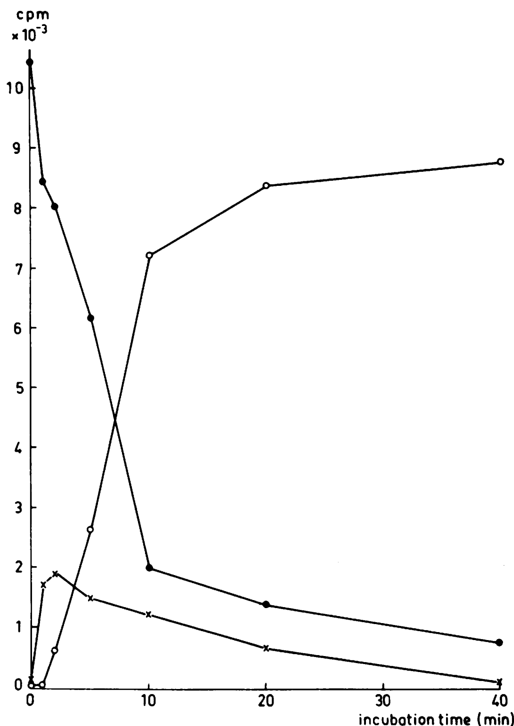

FIG. 5. *Kinetics of the incorporation of UDP-GlcNAc-^{14}C into lipid intermediate and peptidoglycan. Incubation at 37 C was carried out as described in the text. The enzyme was not diluted. After inactivation at indicated times, samples were heated and chromatographed. The activities of peptidoglycan* (O), *UDP-GlcNAc* (●), *and lipid intermediate* (×) *are given.*

(-pentapeptide)-P-P-phospholipid or GlcNAc-MurNAc(-pentapeptide)-P-P-phospholipid (1) was inhibited by enduracidin, moenomycin, prasinomycin, and 11.837 RP, UDP-GlcNAc-^{14}C and unlabeled UDP-MurNAc-pentapeptide were used as substrates. In this case, the radioactivity was also accumulated in the lipid fraction, indicating that these four antibiotics inhibited the utilization of GlcNAc-MurNAc(-pentapeptide)-P-P-phospholipid, the same reaction that was found to be inhibited in the *Micrococcus lysodeikticus* particulate system by vancomycin and ristocetin (1). Autoradiograms, showing the inhibition of in vitro peptidoglycan synthesis by enduracidin, are shown in Fig. 4.

The act: n of the four antibiotics on peptidoglycan synthesis and activity in the lipid fraction was compared to that of vancomycin by incubation in the absence and presence of different antibiotic concentrations (Fig. 6). Vancomycin always gave the least inhibition, whereas enduracidin, moenomycin, prasinomycin, and 11.837 RP were about equally active. The degree of inhibition of peptidoglycan synthesis by the antibiotics was rather irreproducible for unknown reasons. However, when peptidoglycan synthesis was inhibited, the activity in the lipid fraction was always high.

Kinetics of the synthesis of lipid intermediate and peptidoglycan in the presence of antibiotics. A time course of peptidoglycan synthesis showed that the activity of the precursor first appeared in the lipid fraction. The activity of the lipid fraction decreased when UDP-GlcNAc-^{14}C was limiting (Fig. 5). When one of the antibiotics enduracidin, moenomycin, prasinomycin, or 11.837 RP was present during incubation, the rate of synthesis of the lipid intermediate was normal, whereas peptidoglycan synthesis was inhibited. Figure 7 shows a time course from the effects from moenomycin on the activities of peptidoglycan and lipid. Enduracidin, prasinomycin, and 11.837 RP had the same effect. The antibiotics obviously inhibit the utilization of GlcNAc-MurNAc(-pentapeptide)-P-P-phospholipid for peptidoglycan synthesis.

DISCUSSION

In an earlier paper from our laboratory a method was described for the specific incorporation of ^{14}C-L-alanine from a "cell wall medium" into the *E. coli* murein and its alanine-containing precursors (Lugtenberg and de Haan, *in press*). In the present paper, we used this method to demonstrate inhibition of cell wall synthesis in *B. cereus* and *S. aureus* by four antibiotics. Strong inhibition of ^{14}C-L-alanine incorporation was observed (Fig. 2), accompanied by the accumulation of a component with the same chromatographic behavior as UDP-MurNAc-pentapeptide. Vancomycin, known to inhibit murein synthesis, accompanied by accumulation of UDP-MurNAc-pentapeptide (25) gave the same results in both *S. aureus* and *B. cereus*.

The particulate enzyme of *B. stearothermophilus* is very active in peptidoglycan synthesis (26) and was, therefore, chosen to study the effect of the antibiotics on the envelope-bound steps.

The radioactive product formed by the particulate enzyme is peptidoglycan. When one of the precursors was omitted, almost no product was formed (Table 1). Moreover, although only about 70% of the newly formed material could be degraded by lysozyme, this percentage increased to more than 90% when the material was formed in the presence of penicillin (Table 1).

A striking difference between the peptidoglycans synthesized in vitro by the *E. coli* system, as studied by Izaki et al. (15) and by the *B. stearothermophilus* system described in this paper, is the solubility in water and the accessibility

TABLE 2. *Influence of antibiotics on the peptidoglycan synthesis by the Bacillus stearothermophilus particulate enzyme*[a]

Incubation mixture	Additions (μg/ml)	Peptidoglycan (counts/min)	Alanine (counts/min)	Lipid (counts/min)
Complete		1,435	725	171
No UDP-GlcNAc[b]		61	118	43
Heated at zero time		23	8	74
Complete	Bacitracin (5)[c]	50	111	70
Complete	Enduracidin (15)	667	434	352
Complete	Enduracidin (30)	187	509	506
Complete	Moenomycin (15)	189	286	253
Complete	Moenomycin (30)	186	291	313
Complete	11.837 RP (15)	270	429	351
Complete	11.837 RP (30)	183	336	236
Complete	Vancomycin (10)	776	361	217
Complete	Vancomycin (20)	490	299	248

[a] Alanine-labeled uridine diphosphate-*N*-acetyl-muramyl-pentapeptide was the radioactive substrate. The particulate enzyme (P-3′) was diluted 1:1. Incubation was carried out at 37 C for 20 min.
[b] Uridine diphosphate-*N*-acetyl-glucosamine.
[c] Expressed in international units per milliliter.

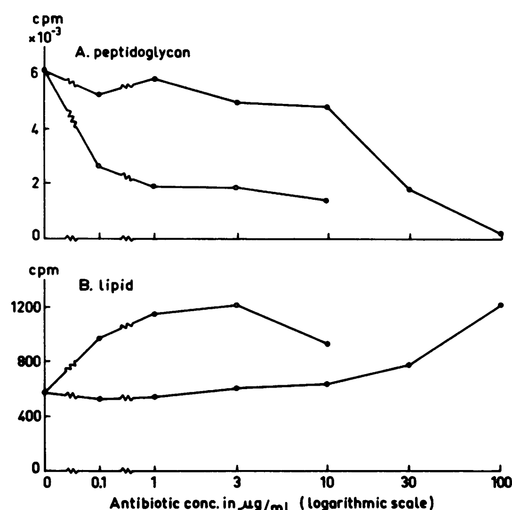

FIG. 6. *In vitro inhibition of peptidoglycan synthesis by different concentrations of prasinomycin* (O) *and vancomycin* (●). *For experimental conditions see Fig. 4. The activities of peptidoglycan* (*A*) *and lipid* (*B*), *measured after 40 min of incubation, are plotted. The curves for enduracidin, moenomycin and 11.837 RP were nearly identical to those for prasinomycin.*

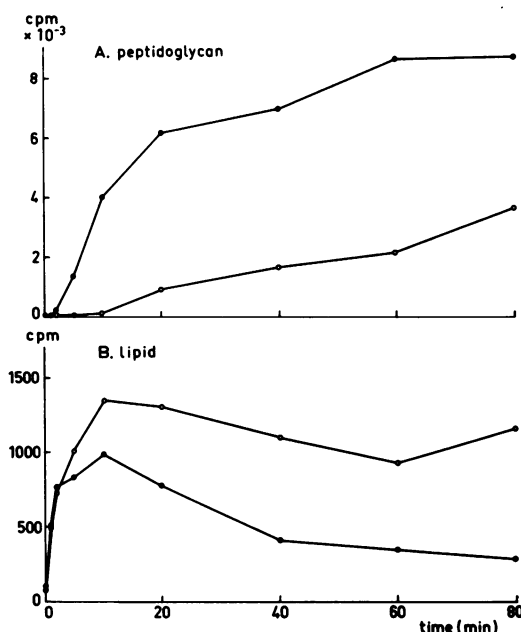

FIG. 7. *Kinetics of inhibition of peptidoglycan synthesis. The complete mixture was incubated in the absence* (●) *and presence* (O) *of moenomycin* (*5 μg/ml*) *at 37 C. At intervals, samples were heated and chromatographed, followed by autoradiography for 4 weeks. UDP-GlcNAc-^{14}C was the labeled precursor. Enzyme from P-3′ was used. The protein concentration in the final mixture was 0.95 mg/ml. The activities of peptidoglycan* (*A*) *and lipid* (*B*) *are plotted against time.*

to lysozyme. The product from the *B. stearothermophilus* enzyme was much less soluble. In both cases, solubility was increased when the incubation mixture contained penicillin (from 9 to 31% in the case of *B. stearothermophilus* and from 50 to 80% in the case of *E. coli*). The peptidoglycan synthesized by the *E. coli* particulate enzyme can completely be degraded by lysozyme (15). The peptidoglycan synthesized by the *B. stearothermophilus* particles is better protected, except when it was synthesized in the presence of penicillin. The fact that the in vitro synthesized peptidoglycan from *B. stearothermophilus* is less soluble and better protected to lysozyme degrada-

tion suggests that the *Bacillus* particles imitate the in vivo situation better than the *E. coli* particles. The *B. stearothermophilus* system is much more active (26), probably because it utilizes GlcNAc-MurNAc(-pentapeptide)-P-P-phospholipid faster, which can be seen when Fig. 3 in reference 15 is compared to Fig. 5 in this paper.

Enduracidin, moenomycin, prasinomycin, and 11.837 RP inhibited peptidoglycan synthesis when tested in the *B. stearothermophilus* cell-free system (Table 2, Fig. 4, 6, 7). This was in good agreement with the observed in vivo inhibition of cell wall synthesis, accompanied by UDP-MurNAc-pentapeptide accumulation. From the results of the in vitro experiments (Table 2, Fig. 4, 6, 7) can be concluded that the synthesis of GlcNAc-MurNAc(pentapeptide)-P-P-phospholipid is not influenced, but that these antibiotics inhibit the utilization of this lipid intermediate (Table 2, Fig. 4, 6, 7). Vancomycin and ristocetin inhibit the same reaction (1, 15). The mechanism of action of vancomycin has been studied rather thoroughly (4–8, 16, 19, 22, 23, 29). The results presented in this paper only show that the new antibiotics inhibit the same step in peptidoglycan synthesis as vancomycin does. A more extensive investigation is required to solve the question whether the mechanisms of action of vancomycin, enduracidin, moenomycin, prasinomycin, and 11.837 RP are identical.

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